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16. ABSTRACT

A variety of graphical and computational methods can be used to derive a median lethal concentration (LC50) from concentration-mortality data produced by an acute mortality test. In the selection of a method, practical considerations should receive as much attention as the usual theoretical and statistical considerations. No method should be used that does not calculate both the LC50 and its 95 percent confidence limits. Unfortunately, several computational methods will not calculate an LC50 from some concentration-mortality data that are practically and statistically useful to aquatic toxicologists. Useful, statistically sound information about the LC50 and its 95 percent confidence limits can be calculated from the data produced by any acute mortality test that meets published criteria of acceptability, by using an internally consistent scheme based on the moving average method and the binomial test.

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Methods for Calculating an LC_{50}

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ABSTRACT: A variety of graphical and computational methods can be used to derive a median lethal concentration (LC_{50}) from concentration-mortality data produced by an acute mortality test. In the selection of a method, practical considerations should receive as much attention as the usual theoretical and statistical considerations. No method should be used that does not calculate both the LC_{50} and its 95 percent confidence limits. Unfortunately, several computational methods will not calculate an LC_{50} from some concentration-mortality data that are practically and statistically useful to aquatic toxicologists. Useful, statistically sound information about the LC_{50} and its 95 percent confidence limits can be calculated from the data produced by any acute mortality test that meets published criteria of acceptability, by using an internally consistent scheme based on the moving average method and the binomial test.

KEY WORDS: water analysis, toxicology, water pollution, moving average, bioassay, probit, biostatistics, toxicity test

Sound experimental toxicology requires competence in biology, chemistry, and statistics. Since most people working in aquatic toxicology are either biologists or chemists who rarely have the services of an experienced biostatistician available on a regular basis, questions often exist concerning both experimental design and analysis of data. This paper discusses some important statistical concepts in one area of data analysis in a reasonably detailed but relatively nonmathematical manner.

The purpose of a toxicity test is to produce data concerning the adverse effect of an agent on test organisms. The most common type of toxicity test with aquatic animals is the acute mortality test which is usually conducted to obtain information about a median lethal concentration (LC_{50}). The data produced by the test generally consist of the percentages of organisms that are killed by different concentrations of a toxicant after specified lengths of exposure. A statistical estimation method is then used to obtain the best estimate of the LC_{50} from the concentration-mortality data for each length

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of exposure. One purpose of this paper is to discuss statistical estimation methods that might be used by aquatic toxicologists and to present a scheme for deriving useful, statistically sound information about the LC_{50} and its 95 percent confidence limits from data produced by any acute mortality test that is acceptable according to the criteria set forth by the Committee on Methods for Toxicity Tests with Aquatic Organisms [*1*].[2] Although only LC_{50} values will be considered herein, the same statistical considerations generally apply to obtaining an estimate of a median effective concentration (EC_{50}), median lethal dose (LD_{50}), or median effective dose (ED_{50}), from quantal (dichotomous, binomial, binary) data, that is, data consisting of two mutually exclusive categories, such as alive or dead.

Since the LC_{50} is the median of a population, whenever an estimated LC_{50} is reported, some measure of the dispersion of the population should also be reported. Common measures of dispersion that could be used are the estimated standard error of the $\log_{10}LC_{50}$, the estimated variance, the estimated relative standard deviation or coefficient of variation, and the upper and lower 95 percent confidence limits (UCL and LCL). The confidence limits are preferred because they are in the same units as the LC_{50} and are therefore most easily understood and used by applied toxicologists and engineers. The 95 percent confidence limits define an interval such that, if all possible replicate 95 percent confidence intervals were determined in the same manner with test organisms from the same population, 95 percent of them would include the true LC_{50} of the population under the conditions of the test. Although fiducial limits are not the same as confidence limits, no distinction between the two is made herein since they are usually numerically equal [*2-5*].

Unfortunately, the confidence limits of the LC_{50} give no information about the precision of the acute mortality test itself. The LC_{50} and its 95 percent confidence limits describe the distribution of the susceptibilities of the individual test organisms. Confidence limits calculated from one test give no indication of the reproducibility or repeatability of the acute mortality test. In order to obtain information about the precision of the acute mortality test, replicate tests must be conducted at different times in one laboratory or in different laboratories [*6,7*].

Necessity for Partial Kills

Some aquatic toxicologists feel that an acute mortality test is not acceptable unless at least one concentration of the toxicant kills some, but not all, of the test organisms exposed to it, and a few believe that two partial kills are a minimum for an acceptable test. Furthermore, some investigators feel that at least one partial kill should be reasonably close to 50 percent, such as between 16 and 84 percent, if a reliable estimate of the LC_{50} is to be

[2] The italic numbers in brackets refer to the list of references appended to this paper.

obtained. A second purpose of this paper is to argue against the necessity for partial kills for most purposes for which acute mortality tests are conducted with aquatic animals.

Aquatic toxicologists who believe in the importance of partial kills usually maintain three assumptions: (*a*) the more partial kills, the better the test, (*b*) the closer the partial kills are to 50 percent, the better the test, and (*c*) the smaller the spread between the 95 percent confidence limits, the better the test. Unfortunately, for tests conducted using the same constant ratio between concentrations, the third assumption contradicts the first two. In this case, the more partial kills, the greater the spread between the confidence limits; and the closer the partial kills are to 50 percent, the greater the spread between the confidence limits.

Let us examine data from some hypothetical toxicity tests. The results in Table 1 were calculated using the probit estimation method described by Finney [5], assuming 20 test organisms per treatment. Using the criteria that (*a*) the more partial kills, the better the test and (*b*) the closer the partial kills are to 50 percent, the better the test, the tests are ordered as to "goodness," with 1 being the best and 9 being the worst, except that Tests 3 and 4 are probably about equally good. On the other hand, using the criterion that the smaller the ratio of the confidence limits (R = UCL/LCL), the better the test, the order of "goodness" is reversed, with 8 being the best and 1 being the worst. Even though the difference in R is not great, the trend is consistent.

This same comparison is also true in Table 2 where, as in Table 1, in every test, each toxicant concentration is 60 percent of the next higher one. Comparison can also be made between the tables. Test 11 has one more partial kill than Test 1, and this partial kill is at 50 percent, the optimum location, but R is larger for Test 11 than for Test 1. This relationship is true for every test in Table 2 in comparison with its counterpart in Table 1. Tables 1 and 2 demonstrate that, if two tests are conducted on two different toxicants using the same ratio between adjacent concentrations and produce different numbers of partial kills, the test which produces more partial kills does not result in closer confidence limits than the test which produces fewer partial kills. Thus one common argument for partial kills is not valid.

A second reason that acute mortality tests producing no or one partial kill(s) are considered unacceptable by some investigators is that some of the more popular statistical estimation methods, such as the unmodified probit and logit methods, require two partial kills. These kinds of estimation methods consist of a transformation, such as probit or logit, and a curve-fitting technique, such as maximum likelihood or minimum chi-square. These are called parametric methods because they assume that the data fit a particular curve and then find the best fit of the curve to the data. Unfortunately, parametric methods cannot calculate an LC_{50} and its 95 percent

TABLE 1—*Results of hypothetical toxicity tests (20 organisms per treatment) using the unmodified probit method* [5].

Test Number	Percent Mortality Concentration of Toxicant, μg/litre							LC_{50}, μg/litre	LCL, μg/litre	UCL, μg/litre	*R*
	462.96	277.78	166.67	100.00	60.00	36.000	Control				
1	100	80	60	40	20	0	0	129.1	104.7	159.2	1.52
2	100	90	60	40	10	0	0	129.1	107.2	155.4	1.45
3	100	90	70	30	10	0	0	129.1	107.9	154.4	1.43
4	100	100	60	40	0	0	0	129.1	110.3	151.2	1.37
5	100	100	70	30	0	0	0	129.1	111.4	149.6	1.34
6	100	100	80	20	0	0	0	129.1	112.8	147.7	1.31
7	100	100	90	10	0	0	0	129.1	115.0	144.9	1.26
8	100	100	95	5	0	0	0	129.1	115.6	144.2	1.25
9	100	100	100	0	0	0	0	...[a]	...	...	...

[a] Cannot be calculated.

TABLE 2—*Results of hypothetical toxicity tests (20 organisms per treatment) using the unmodified probit method* [5].

Test Number	Percent Mortality Concentration of Toxicant, μg/litre								LC_{50}, μg/litre	LCL, μg/litre	UCL, μg/litre	*R*
	597.69	358.61	215.17	129.10	77.460	46.476	27.886	Control				
11	100	80	60	50	40	20	0	0	129.1	100.5	165.9	1.65
12	100	90	60	50	40	10	0	0	129.1	103.3	161.3	1.56
13	100	90	70	50	30	10	0	0	129.1	104.5	159.5	1.53
14	100	100	60	50	40	0	0	0	129.1	106.1	157.0	1.48
15	100	100	70	50	30	0	0	0	129.1	107.4	155.2	1.44
16	100	100	80	50	20	0	0	0	129.1	108.8	153.1	1.41
17	100	100	90	50	10	0	0	0	129.1	110.8	150.4	1.36
18	100	100	95	50	5	0	0	0	129.1	112.2	148.5	1.32
19	100	100	100	50	0	0	0	0	...[a]	...	...	...

[a] Cannot be calculated.

confidence limits for toxicity tests producing no or one partial kill(s) without using a gimmick. Fortunately, other methods can. Therefore, a toxicity test should not be judged unacceptable just because it does not meet the requirements of some estimation methods [*8*]. This should not be construed as a blanket objection to parametric methods. They may well be acceptable methods when they can be used. But the acceptability of a toxicity test should not be judged solely on the basis of one kind of method of calculation.

A third reason why acute mortality tests that produce zero or one partial kill(s) have sometimes been considered unacceptable has been due to a concern about the slope of the regression curve. The importance of the slope in aquatic toxicology has been greatly exaggerated, mainly because many aquatic toxicologists fail to distinguish between toxicity tests and bioassays. Most American aquatic toxicologists have followed the example of *Standard Methods for the Examination of Water and Wastewater* [*9*], and have called toxicity tests "bioassays." As Brown [*10*] has indicated, many scientists recognize a basic difference between a bioassay and a toxicity test. One difference between the two is that the effect used in a bioassay may be either a beneficial effect or an adverse one, whereas toxicity tests, by definition, are concerned with adverse effects. More importantly, the purposes of bioassays and toxicity tests are different. The classical purpose of a bioassay or biological assay is the same as that of a chemical assay or analytical chemical measurement, which is to determine the amount or concentration of a specific chemical entity or constituent [*11*]. Typical assays are the measurement of the amount or concentration of a pesticide, metal, or vitamin. In bioassays, as in many chemical assays, the results of the test on the unknown are calculated on the basis of a standard curve obtained with known standards. The generally accepted best way to show that a bioassay is valid and is not subject to interference is to demonstrate that the slope of the regression curve for the unknown is the same as that of the regression curve for the known, that is, to demonstrate that the two regression curves are parallel. The slopes of the regression curves cannot be shown to be parallel unless at least two partial kills are obtained with both the known and the unknown. Thus, concern about the validity of bioassays results in a concern about parallelism and therefore a concern about partial kills.

This concept of bioassays is apparent in books and articles about bioassay, such as those by Bliss [*2*], Cox [*12*], Cornfield [*13*], Dixon and Massey [*14*], Finney [*15,16*], Mantel and Schneiderman [*17*], and Miller [*18*], even if the definitions used therein do not clearly distinguish between bioassays and toxicity tests. The distinction is more clear if one considers the preference of these authors for analytical dilution assays over comparative dilution assays. Laurence and Bacharach [*19*] make the distinction perfectly clear, as do the majority of publications dealing with the results of bioassays, such as Verrett et al [*20*].

Bioassays can be conducted with aquatic organisms; Saila [*21*], Royer [*22*], and Abedi and Turton [*23*] have used fish instead of analytical chemistry to measure the concentration or amount of a chemical. In addition, bioassays are often conducted with aquatic organisms to compare the potency of an aqueous effluent before and after a waste treatment process and to study pesticide degradation [*24,25*]. The problem with many of these kinds of tests is that, because they may be comparing the biological activity of two different materials that do not necessarily have identical active components, the slopes might not be parallel, and the apparent relative concentration or potency might depend on the end point or species used [*12,15*].

When bioassays are conducted with aquatic organisms, the established principles of bioassays, such as the test of validity based on parallelism, should be followed. Determinations of relative potencies or concentrations are not usually useful if different results can be obtained using different organisms, end points, or conditions. Even relative susceptibilities of different species or of one species under different conditions may depend on the percentage effect used as the end point if the regression curves are not parallel.

Because tests with different purposes are likely to have different requirements in terms of experimental design, data analysis, and interpretation of results [*16*], traditionally, bioassays and toxicity tests have been distinguished because they are two fundamentally different kinds of tests. If a test is conducted to compare relative potencies or concentrations, then it ought to be called a bioassay, and it is important to obtain partial kills. If a test is conducted to obtain information about the adverse effects of an agent on organisms, then it ought to be called a toxicity test because this term most accurately and precisely describes the nature of the test. With toxicity tests, there is no reason to be concerned about parallelism and so there is no reason to obtain partial kills.

Another common argument for the necessity to accurately determine the slope and, therefore, to obtain at least two partial kills in acute mortality tests is the question of extrapolation. Some people want to determine not only the LC_{50} but also another value such as the LC_5 or the LC_{95}. One way to attempt to do this is to determine the slope of a regression curve and then extrapolate. Unfortunately, this simple technique can produce very misleading results [5]. Almost all partial kills occur between 10 and 90 percent kill; however, just because a curve seems to give a reasonably good fit between 10 and 90 percent does not mean that it will give a good fit outside this region. For instance, several possible regression curves are very similar between 10 and 90 percent kill [*3,7,26*] but rather different above and below this region. Rarely, if ever, will enough partial kills be produced in any one test to differentiate between the possible regression curves or to determine if any of them fit the data well below 10 or above 90 percent. The only reliable way of obtaining a good estimate of an LC_2 or LC_{98} is to inter-

polate between data in the region of the desired value. Whenever an LC value is determined by any method, its 95 percent confidence limits should also be calculated to provide information about the precision of the value. Even confidence limits can be misleading though because confidence limits obtained by extrapolation are no better than an LC value obtained by extrapolation.

The preceding has shown that the usual arguments for obtaining two or more partial kills in acute mortality tests with aquatic organisms are not valid. This is important to aquatic toxicologists because it means that tests which produce less than two partial kills do not have to be automatically discarded. Aquatic toxicologists do not have to repeat every test that does not produce at least two partial kills, and they do not have to use numerous concentrations spaced close together in an attempt to get partial kills. A dilution factor of 0.6 seems to be an adequate compromise for acute mortality tests with fish because: (*a*) at least one partial kill will usually be obtained, (*b*) the spread between the confidence limits will usually be reasonably small, and (*c*) six toxicant concentrations will usually cover a wide enough range to give both 24 and 192-h LC_{50} values. This is not to say that one should avoid obtaining two or more partial kills; it is just that there is rarely a strong reason why a special effort should be made to obtain two or more partial kills. If two or more partial kills are obtained, the slope of the regression curve and its 95 percent confidence limits should probably be reported because it might be helpful to someone designing a future toxicity test. Hopefully, these warnings about the pitfalls of extrapolation will discourage the misuse of a reported slope.

Tests producing one or no partial kill(s) do provide useful information about an LC_{50}. For instance, a test consisting of three treatments, a high toxicant concentration in which all of the organisms die, and a low toxicant concentration and control in which none die, definitely provides useful information. By using appropriate statistical techniques, useful and statistically sound information about an LC_{50} and its 95 percent confidence limits can be extracted from acute mortality tests that produce less than two partial kills. Because the statistical estimation methods that can be used to calulate an LC_{50} from concentration-mortality data have different properties, it is important to choose a method carefully. Since most literature comparisons of various estimation methods are based on their use with bioassays, such comparisons may or may not be applicable to toxicity tests.

Statistical Estimation Methods

Statistical estimation methods that have been used with data from acute mortality tests with aquatic organisms include graphical interpolation, and the probit, Litchfield-Wilcoxon, logit, Spearman-Karber, Reed-Muench,

and moving average methods. Graphical interpolation methods, such as that described in Ref *9*, can be used with data that contain one or no partial kill(s), but they do not allow the determination of confidence limits. In addition, graphical methods are subjective to the extent that they depend on human judgment. With some sets of data, most people will get similar results, but with other sets of data, results will differ greatly between individuals.

The probit method [*3,5*] uses the probit transformation and the maximum likelihood curve-fitting technique. This method is very tedious if done manually; it can be done easily only on a computer or minicomputer. It can be used only with sets of data that contain at least two partial kills, unless some data are "adjusted," or unless working probits are misused. The concept of adjusting data, which might more accurately be called "fudging," is to say that, even though 0 or 100 percent of the organisms actually died, for the purpose of calculating results, some other values, such as 5 or 95 percent, will be used.

The Litchfield-Wilcoxon [*27*] method is an approximate probit method [*5*] that was designed to produce the same results as the unmodified probit method, but with less effort. It is simpler to do manually than the probit method, but offers no advantages when a computer is available. This method is somewhat subjective because it is partly graphical. Although it can be made to provide estimates of the LC_{50} and its 95 percent confidence limits with one or no partial kill(s),[3] it should not be used in any cases in which the probit method cannot be used if the results are to be considered statistically valid. Since the Litchfield-Wilcoxon method is supposed to give the same results as the unmodified probit method, when the two differ, the probit method is correct.

The logit method [*8,28,29*] is a parametric method that uses the logit transformation and either the maximum likelihood or the chi-square curve-fitting technique. All versions can be used with one or zero partial kill(s) only if "adjusted" data are used.

The Spearman-Karber method can produce biased estimates in some cases and therefore cannot always be used in a routine manner. This is usually not true, however, for a modified Spearman-Karber method [*30*]. The toxicant concentrations must cover the complete range from 0 to 100 percent kill; therefore, unless some assumed data are used, the method cannot be used with many sets of data that most aquatic toxicologists would consider acceptable. If appropriate formulas are used, this method does not require a geometric series of toxicant concentrations or equal numbers of organisms at each concentration [*30,31*], and often produces about the same results as the probit method [*3,31,32*].

The Reed-Muench method is statistically inferior to the other methods

[3] Marking, L. L., personal communication, 1975.

[3,33], cannot calculate confidence limits, and therefore should not be used.

The moving average method, which is widely used in time series analysis [34], can be used with more sets of data than any of the other methods, without using adjusted or assumed data, and often produces about the same results as the probit method [5]. This method is relatively easy to perform manually and to computerize. Like most of the methods, it assumes that the regression curve is linear. If the toxicant concentrations are in a geometric series, and the same number of test organisms are subjected to each treatment, simplified formulas can be used [6], and, in some cases, tables can be used [35-37] to further reduce the time required. If the simplified formulas cannot be used, general formulas must be used [38]. With no partial kills, the method will provide an estimate of the LC_{50}, but not its confidence limits unless "adjusted" data are used. The moving average method should not be used to calculate any LC other than the LC_{50}.

A number of criteria can be used to compare the ability of the various estimation methods to meet the needs of aquatic toxicologists. (*a*) The only methods that should be used to calculate the best estimate of the LC_{50} are strictly computational methods that can also calculate 95 percent confidence limits. Strictly computational methods will produce identical results whenever the computations are performed correctly, and no LC_{50} should be reported without also reporting confidence limits. (*b*) The method should be just as useful whether or not the toxicant concentrations are in a geometric series and whether or not the complete range from 0 to 100 percent kill is covered. In many toxicity tests with aquatic organisms, the measured, and sometimes the intended, toxicant concentrations do not fall exactly in a geometric series or any other series, either by design or accident, especially when the results are based on a quantity like dissolved copper. (*c*) To be useful in all cases, a method should not require exposing the same number of organisms to each toxicant concentration. (*d*) The use of adjusted or assumed data should not be required for any sets of data. It seems inconsistent to develop a statistical method and then have to adjust data or add assumed data so that the method can be used. Why resort to adjusted or assumed data when statistical methods that can handle the data without the use of such gimmicks are available?

Based on statistical considerations, the graphical interpolation and Reed-Muench methods should never be used. Probit, Litchfield-Wilcoxon, and logit methods should be used only with two or more partial kills. The Spearman-Karber method should be used only if the data include both 0 and 100 percent kill. Thus, based on the needs of aquatic toxicologists and the properties of the common statistical estimation methods, the moving average method is the method of choice. This method can be used to calculate the best estimate of the LC_{50} and its 95 percent con-

fidence limits for all acute mortality tests conducted according to the recommendations of the Committee on Methods for Toxicity Tests with Aquatic Organisms [*1*], except that confidence limits cannot be obtained if there are no partial kills.

A modification of the moving average method is the moving average angle method suggested by Bennett [*38*]. The purpose of using an angle transformation with bionomial data is to improve linearity and to stabilize the variance, thus allowing equal weight to be given to each transformed observation. Harris [*39*] published formulas for 95 percent confidence limits for the moving average angle method, but unnecessarily used "adjusted" data. Harris also assumed that the ratio between adjacent concentrations was constant and that the same number of test organisms was exposed to each treatment. In addition, it has been shown that some angle transformations are better than the one used by Harris [*28,40–44*].

Whenever any method is used to analyze concentration-mortality data, whether or not a transformation such as probit, logit, or angle is used on the mortality data, the logarithmic transformation should probably be used on the concentration data. All of the methods assume that the concentration-mortality curve is linear, and it seems to be generally accepted that the curve is more likely to be linear if log concentration is used. Rarely are acute mortality tests with aquatic animals conducted with enough test organisms and enough concentrations close enough together to indicate whether or not the log transformation of the concentration data makes the curve linear. Because evidence indicates that the log transformation is useful for calculating the LD_{50} with nonaquatic animals, it seems prudent to assume that it should be useful for calculating an LC_{50} with aquatic animals. However, some toxic agents, particularly temperature, seem to produce a nearly normal, rather than a log normal, distribution of susceptibilities of test organisms. For these kinds of toxic agents, no transformation should be used with the concentration data. Because individual tests rarely produce enough data to determine if one transformation is more appropriate than another, it is useless to check data from individual tests for linearity to determine what transformation to use. It is better to adopt a consistent approach that seems to be generally appropriate based on the available data [*45*].

Almost all concentration-mortality data produced by acute mortality tests conducted with aquatic animals following the recommendations of the Committee on Methods for Toxicity Tests with Aquatic Organisms [*1*] can be analyzed to produce a statistical best estimate of the LC_{50} and its 95 percent confidence limits using the moving average method and log transformation of the concentration data. The exception is the case of no partial kills, for which the method will calculate an LC_{50}, but not confidence limits. With no partial kills, an estimate of the LC_{50} can also be calculated as $LC_{50} = (AB)^{1/2}$, where A = highest toxicant concentration in which none of the test organisms died, and B = lowest concentration in which

all of the organisms died. If the log transformation is not used, the formula $LC_{50} = (A + B)/2$ will give the same result as the moving average method.

Binomial Test

Although 95 percent confidence limits cannot be calculated without partial kills, generally they must lie between A and B [37]. Fortunately, the binomial test [46,47] gives the probabilities that a specified or more extreme percentage kill would occur at a particular toxicant concentration if that concentration were the LC_{50}. The binomial test, which is often used as the sign test, is an exact method because it makes no approximations and no assumptions about the data. Since, by definition, the probability of any individual organism dying at the LC_{50} is ½, the probability of all exposed organisms dying at the LC_{50} is $(½)^n$, where n = number of exposed organisms. The probability of all organisms living at the LC_{50} is calculated the same way. The probability of ten out of ten animals dying at the LC_{50} is $(½)^{10}$ or 0.00098. In other words, the LC_{50} will kill ten out of ten organisms only 1 in 1024 times. Thus, one can be 99.9 percent confident that any toxicant concentration that kills ten out of ten organisms is greater than the LC_{50}. Using the same reasoning, the probability that the LC_{50} lies between a concentration that killed all of the organisms and a concentration that killed none can be calculated as $2(½)^n$. Therefore, when no organisms die at concentration A and all of them die at concentration B, an estimate of the LC_{50} can be calculated as $(AB)^{1/2}$, with confidence limits A and B. However, these limits are not 95 percent confidence limits. The confidence level associated with these limits depends on the number of organisms exposed and can be calculated from the formula: confidence level = $100[1 - 2(½)^n]$.

Number of Organisms	Confidence Level
5	93.8
6	96.9
7	98.4
8	99.2

Thus, if six or more organisms are used per treatment, the confidence level represented by A and B is always above 95 percent. Thus these limits must be considered useful statistical information about the LC_{50}. Such two-tailed probability calculations can be made whenever the percentage organisms dead at one concentration is higher than 50 percent and is the same as the percentage organisms alive at a lower concentration. This situation is rare

except when both percentages are zero. The binomial test can provide statistically sound, useful information about the results of toxicity tests in a variety of situations in which no other methods are appropriate.

It must be reemphasized that bounds placed on the LC_{50} using the binomial test are not 95 percent confidence limits even though they give some of the same kind of information as confidence limits. Various means might be used in an attempt to estimate 95 percent confidence limits when there are no partial kills. One might assume a distribution of probabilities, use adjusted data, or assume a maximum slope for the regression curve. However, none of these would be statistically sound. All values between A and B are equally likely to be the LC_{50}. One of the values can be selected as an estimate of the LC_{50} by some appropriate means, but the only statistically sound information that can be derived is the probability that the LC_{50} lies between the bounds.

So far, four major points have been made. (*a*) Whether or not they produce partial kills, properly conducted acute mortality tests do provide useful, statistically sound information about the LC_{50} and its 95 percent confidence limits. All acute mortality tests conducted in such a way that they would be considered acceptable if two or more partial kills were obtained should be considered acceptable if one or no partial kills(s) are obtained. (*b*) Several statistical estimation methods produce very similar results for sets of data with which they all can be used. (*c*) The moving average method can be used to calculate an LC_{50} and its 95 percent confidence limits for more acute mortality tests than any other estimation method without using a gimmick. (*d*) Although 95 percent confidence limits cannot be calculated for an LC_{50} derived from a test that produced no partial kills, statistically sound bounds can be placed on the LC_{50}. On the basis of these four points, the recommended scheme for analyzing concentration-mortality data from acute mortality tests with aquatic animals is (*a*) with one or more partial kill(s), use a moving average method and log concentration, and (*b*) with no partial kills, use either a moving average method or the formula $(AB)^{1/2}$ to obtain an estimate of the LC_{50} and use A and B in place of 95 percent confidence limits if at least six organisms were exposed to each treatment. Regardless of what method is used to obtain an LC_{50} and confidence limits, the results should always be compared with the original concentration-mortality data to determine if they are reasonable.

For those who believe in parametric methods, it should be noted that, for many sets of data, the moving average and probit methods give almost identical results (compare Table 3 with Tables 1 and 2). The major difference concerns the kinds of sets of data for which one method will produce results and the other will not. The probit and other parametric methods, but not the moving average method, can calculate an LC_{50} by extrapolation, that is, when no more than 49 percent of the organisms die in any treatment or when no more than 49 percent live in any treatment other than the

TABLE 3—*Results of hypothetical toxicity tests in Tables 1 and 2 using the moving average method* [6].

Test Number	LC_{50}, μg/litre	LCL, μg/litre	UCL, μg/litre	*R*
1	129.1	105.1	158.5	1.51
2	129.1	107.1	155.6	1.45
3	129.1	108.6	155.1	1.43
4	129.1	110.1	151.4	1.38
5	129.1	111.2	149.8	1.35
6	129.1	113.4	147.0	1.30
7	129.1	117.1	142.3	1.22
8	129.1	120.3	138.6	1.15
9	129.1	...[a]	...	...
11	129.1	102.6	162.4	1.58
12	129.1	103.7	160.7	1.55
13	129.1	104.5	159.6	1.53
14	129.1	106.1	157.1	1.48
15	129.1	107.0	155.8	1.46
16	129.1	108.5	153.6	1.42
17	129.1	111.0	150.1	1.35
18	129.1	112.8	147.8	1.31
19	129.1	115.1	144.8	1.26

[a] Cannot be calculated.

control. However, an LC_{50} calculated from such data would usually be very questionable anyway. On the other hand, the moving average method, but not the parametric methods, can produce an LC_{50} and confidence limits with one partial kill, and an LC_{50} with no partial kills. The probit method can produce an LC_{50} and 95 percent confidence limits in both of these cases only if a gimmick, such as "adjusted" data, is used.

Unfortunately, there is no statistical theory to support or justify the use of adjusted data. Also, there is no statistical basis for deciding what adjustment to use. Probably the most widely used adjustment is that proposed by Berkson [*28,48*]. Fortunately, results calculated with and without using this adjustment usually agree well (Table 4). A major problem occurs when there are no partial kills, such as for Test 26. The confidence limits calculated using the adjustment are unrealistically narrow; if confidence limits are even approximated for this case, they should certainly be no closer than 21.6 and 36.0. The binomial test, but not use of adjusted data, is a statistically sound way of dealing with sets of data containing no partial kills

Control Mortality

A question often raised concerning the calculation of an LC_{50} is how to deal with control mortality. Abbott's formula [5] is a statistically sound way of correcting for control mortality if, and only if, the cause of the control

TABLE 4—*Results of hypothetical toxicity tests (ten organisms per treatment).*

Test Number	Percent Mortality						
	Concentrations, μg/litre						
	100.0	60.00	36.00	21.60	12.96	7.776	Control
21	100	100	100	10	0	0	0
22	100	100	100	70	0	0	0
23	100	100	40	10	0	0	0
24	100	100	70	20	0	0	0
25	100	100	30	20	0	0	0
26	100	100	100	0	0	0	0

Test Number	Without Berkson's Adjustment						With Berkson's Adjustment					
	Probit			Moving Average			Probit			Moving Average		
	LC_{50}	LCL	UCL	LC_{50}	LCL	UCL	LC_{50}	LCL	UCL	LC_{50}	LCL	UCL
21	...[a]	...	...	26.5	24.0	29.3	25.9	21.0	32.0	26.5	23.0	30.6
22	...	...	...	19.5	16.7	22.7	19.7	15.9	24.5	19.5	16.2	23.4
23	35.4	28.5	44.0	36.0	29.7	43.6	35.3	27.7	45.0	36.0	29.0	44.7
24	29.3	23.5	36.4	29.3	24.0	36.0	29.2	22.9	37.3	29.3	23.4	36.8
25	35.2	27.9	44.4	36.0	29.4	44.1	35.2	27.3	45.4	36.0	28.7	45.2
26	...	...	...	27.9	...	...	27.9	23.4	33.2	27.9	25.2	30.9

[a] Cannot be calculated.

mortality does not make the rest of the test organisms more susceptible to the toxicant. This assumption is usually questionable in acute mortality tests with aquatic animals. If the percentage mortality during acclimation or testing exceeds a minimum value, it probably indicates that the test organisms were subjected to some kind of stress that was serious enough to kill them. Such stress would probably make the organisms more susceptible to the toxicant. With proper techniques, most aquatic animals can be held and tested in the laboratory with only minimal mortality. If a species cannot be held and tested without significant mortality, toxicity tests should not be conducted with that species until techniques acceptable to the species have been developed. Abbott's formula cannot make valid the results of a test conducted with unhealthy or stressed organisms. If control mortality is below a specified minimum [*1*], it should be reported along with results of the test, but correction of the LC_{50} for this mortality would seem to be a meaningless exercise. For some sublethal acute toxicity tests, use of Abbott's formula may be appropiate if a percentage of the test organisms consistently shows the effect in the absence of the toxicant. In all other cases, if a significant percentage of the control dies or shows the effect, the results of the test should be considered questionable.

Duplicates

Another common question concerns how to analyze data when duplicate test chambers are used for each treatment. Many people simply pool the data from the duplicates. This assumes that the data are homogeneous and makes no use of the duplicate data as such. Why bother to set up duplicates if they are not going to provide any more information than if all the test animals were exposed in the same chamber? One possibility is to examine the data from duplicate test chambers using Fisher's Exact Test [*46,47*] to determine whether or not the duplicate data are homogeneous. Obviously any duplicates that produced the same percentage dead are homogeneous. If the data for any duplicates are not homogeneous, then between-treatment comparisons and the validity of the LC_{50} are suspect. A second possibility, if the duplicates are not totally randomized but are separately randomized in two different rows or two different portions of the same row, is to separately calculate an LC_{50} and 95 percent confidence limits for each set of duplicates and then compare the two sets of results. A third alternative is sometimes possible when a probit or similar method is used. The data for each duplicate can be entered separately and the chi-square for linearity will indicate whether or not the data are heterogeneous, although heterogeneity can be caused by something other than lack of agreement between duplicates.

Concentration Variability

Another interesting problem concerns the fact that the 95 percent confidence limits are calculated only on the basis of biological variation; no error or variability associated with the toxicant concentrations is taken into account. The assumption is made that no error or variability is associated with the concentration, but this assumption is obviously false. Test solutions cannot be prepared for static tests without variation between duplicates. And repeated measurements of the concentration will show variability. The concentration of toxicant in a test solution cannot be known without error. The assumption is even more obviously false for flow-through tests. The variability of toxicant metering systems devices often ranges from 1 to 5 percent. Measured toxicant concentrations will vary more due to analytical error.

Should this source of variation be taken into account in calculation of the results of acute mortality tests? Technically, it should [*7*], and this problem has been dealt with by Acton [*44*] and Patwary and Haley [*49*]. Practically, it may not be worth the effort. Taking into account another source of variation will change the 95 percent confidence limits, but not the LC_{50}. In addition, biological variation should normally be much greater than variability in toxicant concentration [*5*]. If the variability in concentration exceeds certain limits [*1*], the test should be considered invalid anyway, since then the LC_{50} will be in doubt because the organisms were not exposed to even a nearly constant concentration of toxicant. Obviously, variation associated with the toxicant concentrations should be taken into account in the calculation of the 95 percent confidence limits, but at this stage it would seem to be an unnecessary refinement.

Summary

Several benefits would result if aquatic toxicologists were to adopt a uniform way of analyzing data from acute mortality tests. When a variety of aquatic toxicologists were asked to calculate LC_{50} values and 95 percent confidence limits from five sets of hypothetical data, the results were rather variable. Several respondents reported they could not calculate results for data sets that contained less than two partial kills, but several other respondents were able to obtain values. In the determined "ranges of acceptable values" [*1*], the difference between the upper and lower limits was 8, 12, 5, 2, and 13 percent of the midpoint. Thus up to 13 percent variation may be introduced into the results of acute mortality tests just because different methods of calculating the results are used. And these percentages were calculated after several outliers had been discarded. Different results were even received from different people using the same method. For LC_{50}

values calculated for these data using a probit and a moving average method, the maximum was 2.3 percent (Table 4), which is significantly less than 13 percent. A more serious problem is the outliers—the results that are unreasonable in comparison with the concentration-mortality data. Outliers are usually the result of mistakes that occur when manual calculations are performed, programs are written, or data are entered. Thus quality control is at least as important as uniformity. Aquatic toxicologists should attempt to agree on basic principles for calculating LC_{50} values and 95 percent confidence limits from concentration-mortality data so that all investigators obtain similar results from the same sets of data. Toward this goal, formulas, computer programs, and detailed instructions are being developed for using the moving average and some other statistical estimation methods that can be used to calculate an LC_{50} and its 95 percent confidence limits, since the available literature is often unclear, contradictory, misleading, or incomplete. These may be obtained by writing to the author.

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